Final Report for Grant No. DE-FG02-04ER15562
"Maltose Biochemistry and Transport in Plant Leaves"

Final Report

The major question of research funded by this grant is "How are the end products of starch breakdown, in particular maltose and glucose, exported from the chloroplast".

We are addressing this question by several, complementary approaches:

(i) Bioinformatic identification of putative chloroplast envelope membrane transporters in sequenced plant genomes
(ii) Narrowing down the number of candidate genes by comparative genomics (Chlorophytes vs. Rhodophytes) and comparative proteomics (mesophyll vs. bundle sheath cells in corn)
(iii) Heterologous expression of candidate genes in yeast cells and reconstitution of recombinant transporter proteins into lipid vesicles to test substrate specificity
(iv) Physiological and biochemical analysis of Arabidopsis insertional mutants defective in the candidate genes.

In addition, we are developing a novel strategy to study the transmembrane topology of chloroplast envelope membrane transporters that are catalyzing the exchange of metabolites between chloroplast stroma and cytosol.

(i) Bioinformatic identification of putative chloroplast envelope membrane transporters in sequenced plant genomes

We have used the following strategy to identify putative chloroplast envelope membrane transporters: The predicted Arabidopsis proteome was analyzed for proteins containing at least 3 putative transmembrane regions. This subset of proteins was analyzed for the presence of a predicted chloroplast targeting sequence. The resulting subset was analyzed by BLAST searches for proteins having functions other than metabolite transport. The remaining protein set was considered representing putative plastid envelope membrane transporters. This subset was grouped according to the transport classification (TC) system. The results of this analysis have been published (Weber et al., 2005)

(ii) Narrowing down the number of candidate genes by comparative genomics (Chlorophytes vs. Rhodophytes) and comparative proteomics (mesophyll vs. bundle sheath cells in corn)

Comparative genomics of Chlorophytes and Rhodophytes: The rationale for this approach was that red algae (Rhodophytes), in contrast to green plants (Chlorophytes) do not store starch in plastids. Instead, red algal starch (floridean starch) is stored in the

cytosol. We hypothesized that transporters that are involved in the export of starch breakdown products from green plant chloroplasts would not be required in red algae and are therefore not encoded by their genomes. As dataset, we used all currently known green plant genomes and ESTs, and the genomes of the red algae *Cyanidioschyzon merolae* and *Galdieria sulphuraria*. We found that red algal genomes do not encode any of the putative or known green plant chloroplast envelope membrane sugar transporters (Barbier et al., 2005), suggesting that these transporters may play a role in the transport of starch breakdown products. This approach narrowed down the number of candidate genes to four (At5g16150, At1g05030, At5g59250, At5g17520). Of these four, only At5g17250 has a clearly assigned function – it was shown by Alison Smith's group to encode a putative plastid envelope maltose transporter (MEX1; Nittyla et al., Science 2004, 30:87-9). The other three genes encode putative monosaccharide transporters, including the putative plastid envelope membrane glucose transporter pGlcT (Weber et al., Plant Cell 2000, 12(5): 787-802).

Comparative proteomics of corn mesophyll vs. bundle sheath envelope membranes: The rationale for this approach is that transitory starch in maize leaves is exclusively produced and stored in bundle sheath cells, but not in mesophyll cells. Hence, transporters absent in mesophyll cells but present in bundle sheath cells are potentially involved in starch metabolism. As of now, we have purified proteomics-grade chloroplast envelope membranes from mesophyll cells. We have been able to positively identify a large number of putative chloroplast envelope membrane transporters such as the triose phosphate/phosphate translocator, the phosphoenolpyruvate/phosphate translocator, malate and glutamate translocators. However, none of the putative sugar transporters mentioned above could be identified in mesophyll plastid envelope membranes (in proteomics studies of C3 leaf envelope membranes three different putative plastidic sugar transporters have been found; see Weber et al., 2005 for review). Together with the results of comparative genomics (Rhodophytes vs. Chlorophytes) this finding supports the hypothesis that these transporters are involved in starch metabolism.

In an independent approach, we also analyzed publicly available corn microarray data. We found that the maize gene most closely related to At5g16150 (pGlcT) is much higher expressed in bundle sheath than in mesophyll cells, lending support to our proteomics results. For the other candidate genes, currently no microarray data are available. However, analysis of corn ESTs shows that the corresponding genes are likely expressed in leaves. Proteomic analysis of bundle sheath envelope membranes is currently underway.

(iii) *Heterologous expression of candidate genes in yeast cells and reconstitution of recombinant transporter proteins into lipid vesicles to test substrate specificity*

Expression constructs for all four Arabidopsis genes of interest (At5g16150, At1g05030, At5g59250, At5g17520) have been made and transformed into yeast cells. For At516150 and At5g17520 we have established yeast cell lines expressing the transporter proteins, as detected by Western blot analysis using an antiserum directed against a hexahistidine tag that was fused to the proteins of interest (Figure 1). Establishment of yeast cell lines expressing At5g59250 and At1g05030 is currently underway.

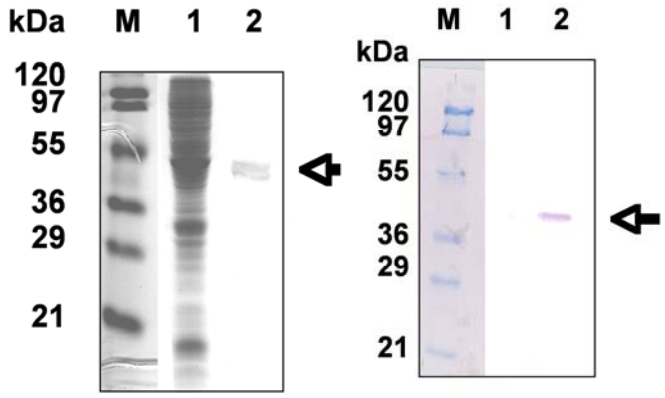

Figure 1. Western Blot of yeast cells expressing hexahistidine-tagged At5g16150 (left panel) and At5g17520 (right panel), respectively.

(iv) Physiological and biochemical analysis of Arabidopsis insertional mutants defective in the candidate genes

Whereas a mutant phenotype has been described for the putative chloroplast maltose transporter (At5g17520, MEX1; Nittyla et al., Science 2004, 30:87-9), the effect of knocking out one or more plastid envelope monosaccharide transporters is currently unknown. This data is required to understand the physiological role of monosaccharide transporters in the plastid envelope membrane. To this end, we have isolated and confirmed at least two independent homozygous knockout alleles for each of the three putative monosaccharide transporters (At5g16150, At1g05030, At5g59250) from the Salk, GABI, and Riken collections. Our previous finding that deletion of At5g16150 results in embryo-lethality could not be confirmed with the additional knockout alleles now available, indicating that the embryo-lethal phenotype was due to either a second site insertion or a larger deletion. We have finished an initial physiological analysis of all knockout lines (presented as a seminar at the 2005 ASPB meeting in Seattle). In short, only a subtle starch excess phenotype could be observed in the lines deficient in At5g16150 whereas no significant effect on starch and soluble sugars could be observed in the other mutant lines. RT-PCR expression analysis of all candidate genes revealed that they show overlapping expression patterns, indicating possible redundant roles in starch metabolism. We have started to generate double- and triple knockout mutants to address this issue. In addition, we have initiated genetic crosses of all individual monosaccharide transporter knockout lines to the maltose transporter mutant MEX1.

Transmembrane topology of chloroplast envelope membrane transporters

The Arabidopsis genome encodes more than 160 putative plastid envelope membrane transporters (Weber el al., 2005), the majority of which has between 4 and 12 transmembrane domains. With the single exception of the triose phosphate/phosphate translocator TPT, nothing is known about the transmembrane topology of these transporters. Information about the transmembrane topology of membrane transporters is

important to gain insight into their biogenesis and folding into their native structure, and for the understanding of structure-function relationships. The study of the transmembrane topology of plastid inner envelope membrane transporters is not trivial due to the presence of an outer envelope membrane that limits the usability of proteolytic and vectorial labeling studies, and the use of domain-specific antisera. We therefore have set out to develop a versatile method for the study of plastid inner envelope membrane proteins (i.e., not limited to transporters) based on functional complementation of a starch biosynthesis deficient Arabidopsis mutant *stf1*.

Experimental strategy:
Translational fusions between the full-length and several truncated versions of a inner envelope transporter with the enzyme phosphoglucomutase (PGM) will be constructed and transformed into the starch free Arabidopsis mutant *stf1* that is deficient in the plastidic isozyme of phosphoglucomutase. Reconstitution of starch biosynthesis (as indicated by a qualitative iodine staining test) by transformation with a particular construct indicates that the C-terminal part of the domain fused to PGM is located in the plastid stroma (see Figure 2). Constructs fused to stroma targeting signals (e.g., Rubisco small subunit) and single transmembrane proteins will be used as controls.

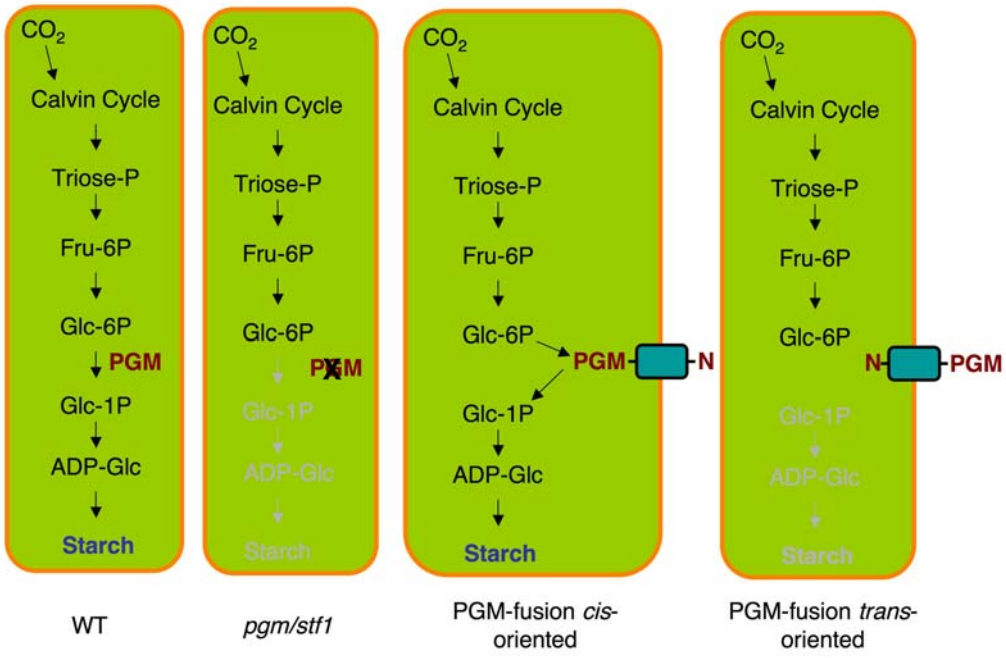

Figure 2. *Schematic representation of a novel strategy to determine the transmembrane topology of plastid inner envelope membrane proteins*. Please note that the outer envelope membrane is not depicted for the sake of simplicity.

As of now, 12 different fusion constructs have been generated (we are using the chloroplast triose-phosphate/phosphate translocator for these proof-of-principle experiments because some information about its topology is available). Four constructs plus positive and negative controls have been transiently transformed into a starch-free mutant of tobacco to test the feasibility of the approach (Figure 3).

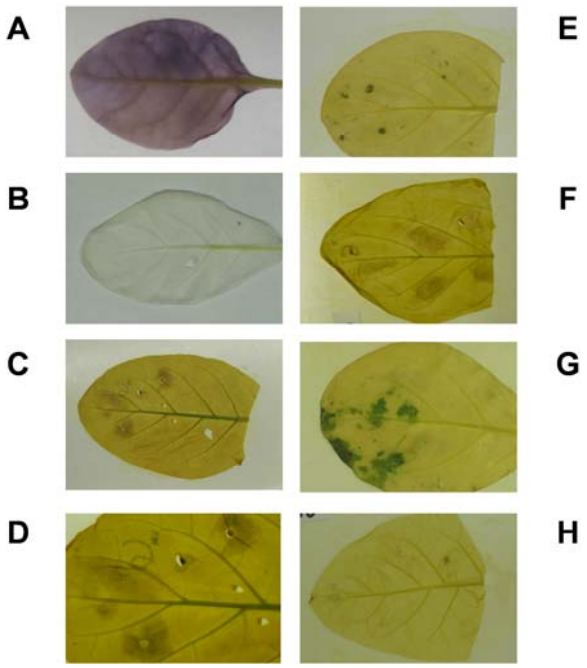

Figure 3. Starch free tobacco mutant deficient in plastidic phosphoglucomutase transiently transformed with phosphoglucomutase fused to truncated version of the plastidic transporter TPT. A: wild type tobacco; B: mutant tobacco line; C: mutant line transformed with authentic plastidic PGM (positive control); D: close-up of C, please note the accumulation of iodine-stained starch in the regions surrounding the infiltration sites; E: mutant transformed with plastidic PGM lacking its transit sequence (negative control); F: PGM fused to the transit peptide of TPT; G: PGM fused to the first transmembrane domain of TPT; H: PGM fused to the sixth transmembrane domain of TPT.

Figure 3 demonstrates the feasibility of the approach, although starch accumulation in the transiently transformed leaf segments is low. Stable transformation into a starch-free Arabidopsis mutant is expected to give stronger signals. The results obtained so far indicate that the TPT transit sequence has stroma-targeting function (starch accumulation, Figure 3F), whereas the N-terminal ends of transmembrane domains one and six are facing the intermembrane space (no starch accumulation, Figure 3G and H; please note the green staining visible in Figure 3 G is due to residual chlorophyll).

Publications resulting from the grant:

Weber, A.P.M. & Fischer, K. (2007) Making the connections - the crucial role of metabolite transporters at the interface between chloroplast and cytosol. FEBS Lett, 581, 2215-2222.

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Bräutigam, A., Gagneul, D. & Weber, A.P.M. (2007) High-throughput colorimetric method for the parallel assay of glyoxylic acid and ammonium in a single extract. Anal Biochem, 362, 151-153.

Reumann S. & Weber A.P.M. (2006) Plant peroxisomes respire in the light: Some gaps of the photorespiratory C2 cycle have become filled - others remain. Biochim Biophys Acta, 1763, 1496-1510.

Bouvier F, Linka N, Isner J C, Mutterer J, Weber APM, Camara B. (2006) Arabidopsis SAMT1 Defines a Plastid Transporter Regulating Plastid Biogenesis and Plant Development. Plant Cell 18, in press.

Weber APM (2006) Synthesis, Export, and Partitioning of the End Products of Photosynthesis. *In* RR Wise, JK Hoober, eds, The Structure and Function of Plastids. Springer, Dordrecht, NL, pp 273-292

Tegeder M, Weber APM (2006) Metabolite transporters in the control of plant primary metabolism. *In* WC Plaxton, MT McManus, eds, Control of Primary Metabolism in Plants. Blackwell Publishing Ltd, Oxford, UK, pp 85-120

Weber APM, Schwacke R, Flügge UI (2005) Solute transporters of the plastid envelope membrane. Annu Rev Plant Biol 56: 133-164

Barbier G, Oesterhelt C, Larson MD, Halgren RG, Wilkerson C, Garavito RM, Benning C, Weber APM (2005) Genome Analysis. Comparative Genomics of Two Closely Related Unicellular Thermo-Acidophilic Red Algae, *Galdieria sulphuraria* and *Cyanidioschyzon merolae*, Reveals the Molecular Basis of the Metabolic Flexibility of *Galdieria* and Significant Differences in Carbohydrate Metabolism of Both Algae. Plant Physiol 137: 460-474

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Weber APM (2004) Solute transporters as connecting elements between cytosol and plastid stroma. Curr Opin Plant Biol 7: 247-253

Sharkey TD, Laporte M, Lu Y, Weise S, Weber AP (2004) Engineering plants for elevated CO_2: a relationship between starch degradation and sugar sensing. Plant Biol (Stuttg) 6: 280-288